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(57) Abstract

The invention is a flow-through bioreactor for the retention and culture of cells in perfused media. The bioreactor is a generally rectangular vessel with inlet and outlet ports in the lid allowing for media flow along the longitudinal axis of the vessel. The inner surface of the bottom wall of the bioreactor has a plurality of generally rectangular grooves having a length, a depth, and a width. The grooves are positioned in the bottom wall such that their length is transverse to the longitudinal axis of the vessel, allowing media flow across the width of the grooves. Cells settle into the grooves, where they proliferate and differentiate, without entering the bulk flow of media through the vessel, thus avoiding loss of cells due to media flow. The invention also provides a method for the perfusion culture of hematopoietic cells whereby a suspension of either unselected hematopoietic mononuclear cells or CD34+ selected cells is placed in the bioreactor and cultured without loss of non-adherent stem/progenitor cells. Various cytokines can be added to the culture medium such that the cells in the grooves form colony-forming units (CFU-GM, BFU-E, CFU-Mix), long-term culture initiating cells (LTC-IC), and granulocytic precursors (blast cells, promyelocytes, myelocytes, metamyelocytes).

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FLOW-THROUGH BIOREACTOR WITH GROOVES FOR CELL RETENTION

Technical Field

The present invention is in the field of perfusion culture of cells. The invention apparatus and method involve a bioreactor which allows for flow-through of media while retaining non-adherent as well as adherent cells within the bioreactor chamber. The invention is especially suitable for the culture of hematopoietic cells.

Related Technology

In cell culture, it is often desirable to maintain cells _in vitro_ for an extended time, during which the cells produce waste, acidify the medium, and use up nutrients from the medium. The exhaustion of the medium is accelerated when the cells proliferate and/or differentiate into highly metabolic cell types. Thus a central problem in cell culture is providing a means to refresh the culture medium without disturbing the cells.

Cell types which adhere to the surface of a culture flask may have their media exchanged or refreshed by simply pouring off the spent media and pouring in fresh media. Alternatively, a portion of the spent media may be gently drawn off and replaced with fresh media. Perfusion or flow-through of fresh media may be desirable for the growth of adherent cell types which require frequent or constant refreshment of culture media. However, even adherent cells may be adversely affected by the shear stress inflicted by the bulk flow of media. Adherent cells may be forced away from their moorings by the bulk media flow, and then lost from the culture system. Alternatively, adherent cells may stay attached to their substrate, but be adversely affected by the force of the fluid such that they fail to proliferate and/or differentiate. Part of the adverse effects of perfusion cultures may be attributed to dilution

and wash out of factors produced by the cells themselves, when those factors are necessary for cell development.

Cell types which do not adhere to surfaces, but rather grow in suspension, present an extra challenge for media exchange. The problem is to exchange the media without losing a high proportion of the cells in the spent media.

Non-adherent cells may be retained in bioreactors with the use of physical barriers. A physical barrier may be in the form of a membrane that creates a barrier to the passage of cells, but allows the diffusion of nutrients and metabolic byproducts.

Hollow fiber bioreactors work on the principle of physical barriers. In a hollow fiber bioreactor, the cells are retained behind a semi-permeable membrane (i.e., the fiber material). A typical hollow fiber unit contains thousands of individual hollow fibers. Commonly, the cells are cultured in the spaces surrounding the fibers. Culture media is perfused through the spaces, and metabolic byproducts diffuse through the semi-permeable membrane, into the hollow fibers, and then out of the system. Examples of hollow fiber bioreactors are disclosed in WO 91/18972 (Knazek) and WO 92/10564 (Culver).

Other types of bioreactors are based on the use of semi-permeable membranes or supports (U.S. 5,264,344 (Sneath) and U.S. 5,223,428 (Rose).

The roller-bottle type of bioreactor is designed for even distribution of medium throughout the cell population. Traditionally, cells adhere to the inner surface of the bottle, which is constantly rotated to bathe the cells. Certain roller-bottle bioreactors have increased inner surface area provided by support strips or corrugations

(U.S. 5,010,013 (Serkes); EP 345 415 (Tyndorf); U.S. 3,853,712; U.S. 5,270,205 (Rogalsky); U.S. 5,256,570 (Clyde)).

Other types of bioreactors, known as stirred bioreactors, often include the use of spin-filters and settling tubes in order to retain cells (U.S. 4,760,028 (deBruyne); U.S. 4,906,577 (Armstrong)). Anchorage-dependent cells may be grown on microcarrier beads, which are commonly used in stirred bioreactors (EP 046,681 (Tolbert); U.S. 5,002,890 (Morrison)).

Several types of static culture flasks make use of corrugations, ridges, or bristles on their internal surfaces in order to provide increased surface area for the growth of anchorage dependent cells (U.S 5,084,393 (Rogalsky); U.S. 5,272,084 (O'Connell); U.S. 5,151,366 (Serkes)). U.S. 4,939,151 (Bacehowski) discloses a cell culture bag having a non-smooth inner surface to prevent the inner surfaces from sticking together during manufacturing and sterilization processes. A three-dimensional solid matrix has also been proposed for growing adherent cells (US 4,514,499 (Noll).

Researchers have had the most experience to date culturing certain specific types of cells, including bacteria, antibody producing hybridomas, fibroblasts, and eukaryotic cell lines. Other types of cells, such as hematopoietic cells, present unusual challenges in the design of a suitable bioreactor.

For certain cancer treatments, it is desirable to culture hematopoietic cells in order to administer the cultured cells to a patient. Hematopoietic cells are obtained from a donor's or a patient's bone marrow or peripheral blood.

The starting cell suspension to be cultured may contain a variety of hematopoietic cells in various stages of differentiation. Alternatively, the cell suspension may first be subjected to certain selection processes, resulting in a starting cell sample highly enriched for stem cells, for instance. Stem cells are primitive hematopoietic cells which have the potential to differentiate into cells of all hematopoietic lineages, including granulocytes, lymphocytes, erythrocytes, and megakaryocytes. It is generally believed that stem cells require adherence to a substrate in order to proliferate and develop to a progenitor stage. However, the cells that have progressed to the progenitor stage, and beyond, are thought to be generally non-adherent because their <u>in vivo</u> micro-environment would be a moving fluid (blood), and they would not be adapted for adherence to a static surface. Thus a culture of hematopoietic cells may contain a variety of different cell types including adherent and non-adherent cells. To further complicate the picture, some of the non-adherent cells may adhere to other cells which, in turn, adhere to a surface.

Hematopoeitic cells present additional challenges because they are shear sensitive. Hematopoietic cells do not appear to grow well when suspended in spinner flask cultures. In attempts to provide a micro-environment conducive to hematopoietic cell growth, growth surfaces have been provided with stromal layers. The stromal layer is generally selected to mimic the extracellular matrix in the bone marrow and consists of proteins such as collagen and fibronectin. Bioreactors which depend on the use of stroma are disclosed in WO 90/15877 (Emerson), WO 92/11355 (Emerson), EP 0 358 506 (Naughton), US 5,160,490 (Naughton), and US 4,963,489 (Naughton).

The use of stroma is disadvantageous for several reasons. First, it is time consuming to produce the stromal layer on a cell culture surface, and great care must be taken not to introduce contaminants into the culture vessels. Certain techniques for laying down stroma require the use of living cells, such as fibroblasts, which are different from the cell type to be cultured. The introduction of foreign cell types into a culture vessel complicates the task of culturing a hematopoietic cell suspension suitable for clinical use.

Accordingly, a primary object for this invention is to provide a bioreactor which allows for the exchange of media without undue perturbation or loss of the cultured cells.

Another object for this invention is to provide a bioreactor which permits retention of cells without the use of stroma.

Another object of this invention is to provide a flow-through bioreactor which permits cultured cells to be easily and efficiently recovered from the bioreactor chamber.

A further object of this invention is to provide a method for the perfusion culture of hematopoietic mononuclear cells, unselected for CD34+.

These and other objects and advantages of the present invention will be apparent from a reading of the following detailed description of exemplary preferred embodiments of the invention, taken in conjunction with the appended drawing Figures, in which the same reference numeral refers to the same feature throughout the drawing Figures, or to features which are analogous in structure or function.

Dimensions of the grooves are identified as X, Y, and Z. The longitudinal axis of the entire bioreactor vessel is identified as L.

Brief Description of the Drawing Figures

Figure 1 provides a partially schematic front quarter perspective view of a flow-through bioreactor with grooves for cell retention, according to the present invention.

Figure 2 provides a longitudinal cross-sectional view of the bioreactor of the invention.

Figure 3 provides a cross-sectional view through the inlet port and along the length dimension of a groove.

Figure 4 provides enlarged fragmentary cross-sectional views of the grooves. Figure 4a shows a groove of one embodiment of the invention, in which each groove has a ratio of width:depth = 1:1. Figure 4b shows a groove of a different embodiment of the invention, in which each groove has a ratio of width:depth = 2:1.

Detailed Description of Exemplary Preferred Embodiments of the Invention

Viewing Figure 1, the bioreactor vessel 10 is shown with the lid 12 expanded from the view of the receptacle 14, in order to show the details of the inner surface of bottom wall 16. In operation, the lid 12 is sealed to the receptacle 14 by means known in the present art. For instance, the lid 12 may be permanently sealed to the receptacle 14 by means of chemical bonds, or may be sealed by means of a gasket and clamp. Alternatively, the entire bioreactor vessel 10 may be molded in one piece. Preferably, the bioreactor vessel 10 is made of a clear

plastic material such as polycarbonate, polysulfonate, acrylic, or polystyrene. The inner surface of the vessel 10 may also be coated with teflon or another polymer, or may have a negative charge added, according to the growth requirements of the particular cell type to be cultured.

The inner surface of bottom wall 16 is provided with a plurality of long rectangular grooves 18 in which cells are retained while culture medium flows along the longitudinal axis L of the receptacle 14, in a direction transverse to the length dimension X of the grooves 18. Grooves 18 are disproportionately enlarged in this figure for better illustration.

The lid 12 has an inlet port 20, for conveying liquid media through inlet slot 22. The media flows from inlet slot 22, along the longitudinal axis L through the bioreactor vessel 10, and out the outlet slot 24. Outlet slot 24 connects with an outlet port (26 in Figure 2). The media flow is regulated by well known means such that the flow is even across the inner surface of bottom wall 16. One example of means to regulate flow is provided in experimental Example 1 below.

Figure 2 provides a longitudinal cross-sectional view of above described elements: inlet port 20, inlet slot 22, outlet slot 24, outlet port 26, inner surface of bottom wall 16, grooves 18. In this figure, grooves 18 are disproportionately enlarged for better illustration, and groove detail has been omitted on portions of inner surface of bottom wall 16. However, in the preferred embodiment of the invention, grooves 18 are continuous across the inner surface of bottom wall 16.

Inlet port 20 is connected to a reservoir of fresh media which is maintained at a suitable physiological pH by means well known in the art of cell culture. Outlet port 26 may be shunted to a waste container, or the media exiting outlet port 26 may be refreshed by well known means and recirculated to inlet port 20.

Figure 3 is a cross-sectional view of bioreactor 10 in dimension X (Figure 1), through inlet port 20 and inlet slot 22. This sectional view runs the length of a groove 18, showing the length face 30 of a groove 18.

Figure 4a is a cross-sectional view, perpendicular to dimension X (Figures 1 and 3), showing the dimensions of a groove 18 in one preferred embodiment of the invention. In this embodiment, the ratio of width Y to depth Z is about 1:1. Suitably, width Y and depth Z are each about 50μm to about 5,000μm. Preferably, width Y is about 200μm and depth Z is about 200μm. Using dimensions Y:Z=200μm:200μm, a monolayer of hematopoietic cells 32 (approximately 10μm deep), resting on the groove bottom 34, would change the groove width to depth ratio by only about 5%.

Although the groove 18 is depicted with corners and edges forming sharp 90° angles, it is understood that within the scope of this invention, corners and/or edges of the grooves might be rounded to form arcs. Given the present disclosure, it is also understood that different types of groove geometries may be devised to achieve similar results.

Figure 4b shows the dimensions of a groove 18 in a second preferred embodiment of the invention. In this embodiment, the ratio of width Y to depth Z is about 2:1.

The preferred groove dimensions are suitable for retention of cells 32, both adherent and non-adherent, when media flows along longitudinal axis L (see Figure 1) over the inner surface of bottom wall 16, across the top of the groove 18 (Figure 4). As will be demonstrated in experimental examples below, the bulk flow of media along longitudinal axis L over the inner surface of bottom wall 16 does not perturb cells 32 within the grooves 18. Both adherent and non-adherent hematopoietic cells are able to proliferate and differentiate in the grooves 18 of the bioreactor of the present invention. Under regulated flow conditions, there is no appreciable loss of cells due to wash out. The fact that the cells thrive demonstrates that nutrients, growth factors, and oxygen from the bulk flow of fresh media across the mouth of the grooves 18 enter the fluid in the grooves to maintain the cells. Moreover, the health of the cultured cells indicates that the cells' deleterious metabolic by-products such as CO_2 diffuse out of the fluid in the grooves 18, into the bulk flow of media across the inner surface of the bottom wall 16, and ultimately out of the bioreactor. Moreover, the fact that essentially no cells are lost indicates that the cells themselves do not exit the mouths of the grooves 18 to enter the bulk flow along the longitudinal axis L of the bioreactor vessel.

One might be led to consider whether the success of the bioreactor of the present invention could be partially explained by theoretical flow patterns (Higdon, J.L., 1985, J Fluid Mech 159:195-226; Chilukuri, R., et al., 1984, J Electrochem Soc 131:1169-1173; Tighe, S., et al., 1985, Chem Eng Commun 33:149-157; Chilukuri, R., et al., 1983, Chem Eng Commun 22:127-138). Without the complication of cells in the grooves, the external flow across the inner surface of the bottom wall 16 might be incapable of

penetrating the small grooves 18 in the surface, and thus might not displace the media from the grooves. Also, without cells in the grooves, a circulatory flow or "eddy" might be induced within each groove such that dissolved nutrients and gases might be exchanged by diffusion between the media in the grooves and the media in the external flow. However, the presence of cells in the grooves renders theoretical predictions of flow impractical within the present state of the art of fluid dynamics.

Flow patterns within the working bioreactor of the present invention cannot be practically described using theoretical calculations. However, this does not diminish the importance of the discovery of the present bioreactor that permits the retention, proliferation, and differentiation of non-adherent cells as well as adherent cells. The application of the bioreactor of the present invention to the culture of hematopoietic cells will be described in the experimental examples below.

A particular advantage of the method of the present invention is that a suspension of hematopoietic mononuclear cells may be successfully cultured without first selecting for CD34+ stem/progenitor cells. To obtain a mononuclear cell suspension, the donor's or patient's blood sample is obtained using a well-known apheresis procedure. For example, the apheresis procedure may be conducted using the Baxter CS-3000™ apheresis machine, or the like. In some cases, the apheresis product is used directly without further processing. In other cases, when visual inspection of the apheresis product indicates the presence of a large excess of red cells, the apheresis product is subjected to density gradient separation to remove most red cells, platelets, and cell debris from the mononuclear cell suspension. The mononuclear cell suspension is placed

directly into the grooved bioreactor and cultured in perfused media. Herein, the terms "mononuclear cells" and "mononuclear cell suspension" refer to hematopoietic cells which have been separated from most red blood cells, platelets, and multinucleated granulocytes. It is understood that the mononuclear cell suspension contains a very small fraction of CD34+ stem/progenitor cells. The culture method of the present invention allows the propagation and differentiation of the small number of stem/progenitor cells within the starting suspension, without disadvantageous media depletion by the numerous mature cell types in the suspension.

EXAMPLE 1

<u>Culture of Peripheral Blood Cells in Flow-Through Grooved Bioreactor Compared With Stroma and Static Culture.</u>

Perfusion cultures in the grooved bioreactor were compared with perfusion cultures on a stromal layer (no grooves). Control static cultures were performed in either a smooth surfaced flask (no grooves) or a flask with a stromal layer.

Methods: Peripheral blood cells were obtained from two clinical sources. These cells were "mobilized" from the bone marrow of cancer patients into their peripheral blood by treatment of the patients with chemotherapeutic agents and cytokines, and collected by apheresis. The cells were received by overnight shipment in RPMI-1640 with 5% serum either on ice or at room temperature. The mononuclear cells were obtained by Ficoll density gradient (1.077 gm/cm3) centrifugation (1200 rpm for 20 minutes). The mononuclear layer obtained was washed once with 1 X Ca++ Mg++ free phosphate buffered saline (PBS). The peripheral blood mononuclear cells used in the bioreactor studies had between 1 and 3% CD34+ cells (stem cells).

Culture Media & Growth Factors: Human long term media (HLTM) is composed of McCoy's 5A medium supplemented with 1% MEM Vitamins, 1% 2 mM glutamine, 1% 1 mM sodium pyruvate, 1% MEM essential amino acids, 1% MEM amino acids, 1% 1M HEPES, 1% 10 mM monothioglycerol, 0.1% 50 mg/ml gentamicin sulfate (Gibco), 12.5% preselected heat inactivated fetal bovine serum and 12.5% preselected heat inactivated horse serum. Colony assay medium is composed of 0.8% methylcellulose in IMDM supplemented with 50 μg/ml gentamicin sulfate, 30% preselected heat inactivated fetal bovine serum, 2% bovine albumin (Armour Pharmaceuticals), 150 U/ml recombinant human interleukin 3 (rhIL-3, R&D Systems, Inc.), 40 ng/ml recombinant human interleukin-6(rhIL-6, Sandoz or R&D Systems, Inc.), 150 U/ml recombinant human granulocyte colony-stimulating-factor (rhG-CSF, Immunex), 200 U/ml recombinant human granulocyte-macrophage colony-stimulating-factor (rhGM-CSF, Immunex), and 10 U/ml recombinant human erythropoietin (rhEpo, Amgen). Growth factor supplemented HLTM using the bioreactor studies contained 150 U/ml rhIL-3, 40 ng/ml rhIL-6, 150 U/ml rhG-CSF and 50 ng/ml stem cell factor (SCF, Amgen). All of the reagents were obtained from Sigma unless otherwise specified.

Stroma: Bone marrow cultures were established as reported by Koller et al (Exp Hematol 20:264-270, 1992). Briefly, stromal cells subcultured from 2-week-old marrow cultures were used to form stromal feeder layers by inoculating into 3.75 X 7.5 cm rectangular polycarbonate dishes (Cole Parmer, Chicago IL) at 4 X 10^5 cells/ml in 5 ml HLTM. Each dish contained a 3.75 X 7.5 Thermanox® slide (Nunc, naperville, IL) which served as the culture substratum. After a 24 hour incubation at 37°C in 5% CO_2 in air, dishes were irradiated with a dose of 12 Gy from a ^{137}Cs source.

The following day, cells to be cultured were seeded onto the irradiated stroma for static culture experiments. For stromal bioreactor experiments, the slides coated with stroma were rinsed and placed on the inner bottom surfaces of bioreactor vessels without grooves.

Bioreactor cultures. The culture chambers were constructed of polycarbonate plastic, the tubing and connectors were constructed of Teflon, and the tubing used in the peristaltic pump was made of silicone. The culture chambers had the following dimensions:

L:	Chamber length:	3.00 in or	7.62 cm
W:	Chamber width:	1.50 in or	3.81 cm
H:	Chamber height:	0.21 in or	0.53 cm
Af:	Flow cross section (H W)	0.32 in^2 or	2.03 cm^2
V:	Chamber volume (H L W)	0.95 in^3 or	15.5 cm^3

The grooved bioreactors of the present invention also had the following dimensions:

Y:	Groove width:	200μm
Z:	Groove depth:	200μm

All of the bioreactor parts were washed, sterilized, and reused except for the pump tubing. It is understood that, for clinical use, the bioreactor would be a single-use disposable. The sterile bioreactor was completely assembled in a 37°C incubator (Stericult, Forma Scientific). The culture chambers were placed in a rack that kept the chambers at a uniform 10° angle from horizontal to encourage air bubbles to leave the system. HLTM was then circulated through the bioreactor to allow calibration of the pH and dO_2 probes. For these calibrations, the bioreactor was first equilibrated with CO_2 for the first point of the pH calibration. Second, the bioreactor was equilibrated with air for the second point of the pH calibration and for the dO_2 calibration. The bioreactor was then drained and injected with 30 ml of HLTM and 60 ml of HLTM supplemented with 2X growth factors and the pH controller set at 7.35 ± 0.05. The media was almost entirely drained from the three culture chambers per bioreactor prior to the seeding of the cultures. The

cultures were seeded by injecting 10.0 ml of 2 x 10^5 cells/ml mononuclear cell suspension injected each of the three chambers for each bioreactor. The cells were allowed to settle for 15 minutes, and then the pump was started at approximately 0.2 ml/min. and increased every 15 minutes to 0.5, 1.0, 1.5, 2.0 and finally 2.5 ml/min, At the same time, static control cultures were established in 100 mm polycarbonate petri dishes containing 20 ml of HLTM supplemented with the same growth factors. The bioreactors were fed 3X/week by the replacement of one-half of the culture media. The static cultures were fed every 5 days. One of the three weekly feedings for the bioreactors occurred at the same time as the static cultures, that is when a portion of the cultures were harvested.

The bioreactor flow rate was measured during each feeding and the pH measured with an external pH probe (Corning). Cell counts were performed on the media removed from the cultures using the Coulter Counter (Coulter Electronics). One chamber per bioreactor and one corresponding control culture was harvested on days 5, 10 and 15. The bioreactor cultures were harvested by draining the contents, rinsing once with 10 ml of phosphate buffered saline (PBS), rinsing once with 1 X cell dissociation solution (Sigma), and the rinsing a second time with PBS. This was accomplished in the same manner as for the washout experiments. The control cultures were harvested with the same draining and rinsing schedules. The cell number remaining in the culture vessels was estimated by rinsing once with 10 ml of cetrimide and counting nuclei with a Coulter Counter. The harvested cell suspensions were concentrated by centrifugation (15 minutes at 1200 rpm) and resuspended in approximately 10 ml of fresh HLTM. Cell counts were performed with both a Coulter Counter and also a

hemacytometer. The viability was determined by trypan blue dye exclusion during the hemacytometer counts.
Colony assays were established at 1,000, 3,000, and 9,000 cells/ml for mononuclear cells and 500, 1,500, and 3,000 cells/ml for CD34+ cells. These assays were cultured at 37°C in a fully humidified incubator with an atmosphere of 5% CO_2, 5% O_2 and the balance N_2. Colonies were scored using a 40 X stereomicroscope (Nikon) on day 14. White colonies containing >50 cells were scored as colony-forming-units granulocyte-macrophage (CFU-GM), red colonies containing >50 cells were scored as burst-forming-unit erythroid (BFU-E), and mixed red and white colonies containing >50 cells were scored as colony-forming-units mixed (CFU-Mix).

Long-term culture initiating (LTC-IC) assays were established in 24-well tissue culture plates (Falcon) containing 1×10^5 irradiated (2,000 rad) allogeneic human bone marrow cells per well. The cells being assayed were seeded at 5×10^4 and 2×10^5 cells per well for the harvested mononuclear cells or 2.5×10^4 and 1×10^5 cells per well for the harvested CD34+ cells. Each well contained 2.0 ml of HLTM. The cultures are incubated at 33°C in a fully humidified incubator with an atmosphere of 5% CO_2, 5% O_2 and the balance N_2. The cultures are feed once per week by the replacement of one-half of the media with fresh HLTM. The cultures were harvested after 5 weeks and colony assays established at 15,000 cells/ml. All colonies scored from these colony assays were considered LTC-IC colonies. Flow cytometry was conducted by staining CD33 (Becton Dickinson)/ CD34, CD11b (Becton Dickinson)/ CD15 (Becton Dickinson), CD11b (Becton Dickinson), and Gly A (Amak, Inc.) and analyzing by flow cytometry (FACSTAR).

The static cultures were fed by the replacement of one-half of the culture media every 5 days. In spite of care taken

in feeding, this inevitably led to the loss of some cells, since most or all of the cells were non-adherent.

Results from 3 series of experiments are shown in Table 1 below:

Key to Table 1

Stroma: number of stromal cells initially seeded for stromal cultures.

PBMN cells: number of peripheral blood mononuclear cells initially seeded in both stromal and non-stromal cultures.

Flo-Grv: The flow-through grooved bioreactor of the present invention.

Flo-Strom: A flow-through bioreactor, without grooves, with a stroma-layered slide on the bottom.

Stat/Smooth: A static control culture, no stroma.

Stat/Strom: A static control culture, with stroma.

Table 1

PBE #9 Summary (n = 3)

Day	Cells	Hemacytometer Cells	± Cells	CFU-Mix Assays CFU-c	± CFU-c	LTC-IC Assay CFU-c	CFU-c
-1	Stroma	1,997,883	± 4,469	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
0	PBMN Cells	2,000,846	± 114,665	16,276	± 5,018	3,522	± 1,390
5	Flo/Grv	1,600,333	± 1,309,649	56,141	± 27,399	750	± 466
5	Flo/Stroma	4,005,917	± 3,719,435	64,934	± 44,772	1,158	± 768
5	Stat/Smooth	1,037,167	± 102,098	59,725	± 25,471	383	± 152
5	Stat/Stroma	1,488,250	± 251,706	95,358	± 69,265	745	± 370
10	Flo/Grv	5,211,500	± 1,911,085	204,840	± 50,367	1,226	± 406
10	Flo/Stroma	5,854,000	± 2,116,989	174,193	± 50,403	575	± 500
10	Stat/Smooth	4,984,000	± 1,312,589	135,262	± 14,930	330	± 146
10	Stat/Stroma	6,304,750	± 2,339,924	189,850	± 118,865	335	± 191
15	Flo/Grv	19,842,500	± 9,268,737	119,116	± 70,766	2,263	± 1,940
15	Flo/Stroma	21,751,167	± 2,985,267	174,705	± 74,522	1,190	± 482
15	Stat/Smooth	14,732,833	± 2,700,869	94,865	± 80,835	389	± 398
15	Stat/Stroma	9,683,750	± 2,283,539	65,351	± 38,100	417	± 502

Day	Cells	CFU-Mix Assay CFU-GM	± CFU-GM	CFU-Mix Assay BFU-E	± BFU-E	CFU-Mix Assay CFU-Mix	± CFU-Mix
-1	Stroma	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
0	PBMN Cells	10,505	± 2,967	5,480	± 3,366	291	± 340
5	Flo/Grv	50,760	± 26,184	5,363	± 5,044	19	± 32
5	Flo/Stroma	61,178	± 42,267	3,685	± 4,990	71	± 123
5	Stat/Smooth	57,187	± 26,577	2,537	± 2,603	0	± 0
5	Stat/Stroma	89,387	± 66,207	5,796	± 5,661	175	± 302
10	Flo/Grv	204,013	± 50,292	827	± 752	0	± 0
10	Flo/Stroma	174,193	± 50,403	0	± 0	0	± 0
10	Stat/Smooth	130,825	± 21,466	4,437	± 7,685	0	± 0
10	Stat/Stroma	187,809	± 121,434	2,041	± 3,535	0	± 0
15	Flo/Grv	119,116	± 70,766	0	± 0	0	± 0
15	Flo/Stroma	174,705	± 74,522	0	± 0	0	± 0
15	Stat/Smooth	94,774	± 80,943	92	± 159	0	± 0
15	Stat/Stroma	65,276	± 38,230	75	± 130	0	± 0

Day	Cells	Hemacytometer Viability	± Viability	Cells	Hemacytometer Viability	± Viability
-1	Stroma	#DIV/0!	#DIV/0!	PBMN Cells	#DIV/0!	#DIV/0!
5	Flo/Grv	79%	± 24%	Flo/Stroma	85%	± 18%
5	Stat/Smooth	91%	± 8%	Stat/Stroma	91%	± 3%
10	Flo/Grv	87%	± 10%	Flo/Stroma	78%	± 26%
10	Stat/Smooth	97%	± 2%	Stat/Stroma	92%	± 3%
15	Flo/Grv	95%	± 1%	Flo/Stroma	88%	± 9%
15	Stat/Smooth	95%	± 4%	Stat/Stroma	93%	± 2%

Cell number: The static cultures and the flow-through cultures contained similar cell and colony numbers up to day 10, during which time the cell numbers were relatively low. At day 15, when the cell numbers were relatively high, the performance of the static cultures dropped and the flow-through cultures excelled. Comparing results from both types of flow-through bioreactors at day 15, the number of cells in the grooved bioreactor was comparable to the number of cells in the bioreactor with stroma (no grooves). These results indicate that for cell retention and proliferation the grooved bioreactor performs as well as a bioreactor with stroma at all time points tested.

Colony-forming units: Cultures from the grooved bioreactor contained a number of granulocyte-macrophage/colony-forming units (CFU-GM) comparable to cultures from the stroma-layered bioreactor at all time points. After day 5, few erythroid cells and few BFU-E were detected in any of the cultures because the cytokine mix in the media was designed to drive granulocyte/macrophage differentiation, and not erythropoiesis.

Long-term colony initiating cells: Cultures from both types of bioreactors contained comparable numbers of long-term colony initiating cells at all time points.

Viability: Cells from both types of bioreactors contained comparable number of viable cells at all time points. The viability of recovered cells was very good, ranging from 79 - 97%.

Media supernatant analysis: Media supernatant samples were analyzed for IL-6, GM-CSF, and tumor necrosis factor-α (TNF-α) concentrations. Minimal differences were observed

in cytokine concentrations between the different cultures. The concentration of Il-6 and TNF- increased in all cultures from about 35 ng/ml and about 25 pg/ml (day 0) to about 50 ng/ml and about 50 pg/ml (days 10-15), respectively. The concentration of GM-CSF increased in the stroma-containing cultures from about 20 pg/ml (day 0) to a maximum on day 5 of about 60 pg/ml before falling to levels below input. The concentration of GM-CSF in the stroma-free cultures fell continuously from about 20 pg/ml (day 0) to about 5 pg/ml (days 10-15). In general, the stroma-containing cultures had a slightly faster increase in cytokine concentrations than the stroma-free cultures. Furthermore, the static cultures had a slightly faster increase in cytokine concentrations than the perfusion cultures.

Media supernatant samples were also analyzed for glutamine, ammonia, glucose and lactate concentrations, and media pH. Glutamine concentrations declined in the perfusion cultures from about 0.9 mM (day 0) to about 0.7 mM (day 15) while the glutamine concentration remained at approximately the input level in the static cultures. In addition, ammonia concentrations increased in all cultures from about 300 M (day 0) to about 600 M (days 10-15). This suggests that most of the small amount of glutamine consumption was due to its degradation at 37°C rather than consumption by cells. However, substantial amounts of glucose were consumed and lactate produced in the stroma-containing, but not stroma-free cultures. Glucose concentrations declined from about 240 mg/dL (day 0) to about 210 mg dL (day 15) and about 120 mg/dL (day 10) for the stroma-containing perfusion and static cultures, respectively. Correspondingly, lactate concentrations increased from about 20 mg/dL (day 0) to about 100 mg/dL (day 15) and about 150 mg/dL (day 15) for the stroma-containing

perfusion and static cultures, respectively. Finally, the media pH was controlled at 7.35 +/- 0.05 for the perfusion cultures, but declined from 7.35 (day 0) to about 7.25 (day 10) and about 6.90 (day 10) for the static stroma-free and stroma-containing cultures, respectively. Other observations in our laboratory suggest that CFU-GM are inhibited at pH below 7.20 (personal communication from Todd McAdams). In these studies, the decline in pH below 7.0 coincided with the decline in CFU-GM from day 10 to day 15 in the static stroma-containing cultures.

EXAMPLE 2

Culture of Unselected Mononuclear Cells and CD34+ Selected Cells in the Grooved Bioreactor.

Within the art of hematopoietic cell culture, it is a general belief that a proportion of CD34+ cells are stem cells which may require adherence to a substrate, or stroma. Therefore, it was of interest to determine whether CD34+ selected cells could proliferate in the grooves of the bioreactor of the present invention. There is no stroma in the bioreactor of the present invention. It is understood that the bioreactor could be formed of different types of plastics, or have plastic surfaces treated such that cells could adhere. However, the bioreactor used in the following experiments was formed of a type of plastic, polycarbonate, which is thought to be non-conducive to cell adherence since its surface is neutrally charged.

Peripheral blood samples were obtained and mononuclear cell suspensions were prepared as described in Example 1 above. Phenotypic analysis of the starting samples by flow cytometry showed that the peripheral blood samples originally contained 2 - 12% CD34+ cells. CD34+ cells were selected from the mononuclear cell suspension by first incubating the suspension with mouse monoclonal antibodies against CD34, which bound specifically to the CD34 cell surface antigen on CD34+ cells. Then paramagnetic beads coated with sheep-anti-mouse antibodies were incubated with the cell suspension. The paramagnetic beads then bound the CD34+ cells via binding of the sheep-anti-mouse antibodies to the mouse antibodies on the CD34+ cells, to form bead/CD34+ cell complexes. The bead/CD34+ cell complexes were then selected from the total cell population by magnetic attraction. After washing, the CD34+ cells were released from the beads by enzymatic digestion with

chymopapain. Results of CD34+ selection are shown in Table 2 below.

Table 2. $CD34^+$ selection performance and yield.

	average [a] ± S.D.	(Min - Max)
% $CD34^+$ of Original Cells	6.31% ± 3.86%	(2.23% - 12.32%)
Viability of Original Cells	93.87% ± 8.21%	(79.09% - 99.63%)
% $CD34^+$ of Selected Cells	90.93% ± 10.50%	(70.76% - 97.54%)
Viability of Selected Cells	98.09% ± 2.10%	(95.83% - 100.00%)
Yield on $CD34^+$ Selection [b]	50.63% ± 33.23%	(8.75% - 98.34%)

[a] Average of six experiments.

[b] Yield on $CD34^+$ selection is the percent of the viable $CD34^+$ cells recovered from the selection divided by the viable $CD34^+$ cells initially present in the mobilized blood sample.

The CD34+ cells were seeded into bioreactor and static control cultures as described in Example 1 above, except none of the cultures had stromal layers.

Experiments using mononuclear cell preparations, unselected for CD34+ cells, were conducted in parallel with the CD34+ experiments.

Human long-term medium (HLTM) containing 12.5% fetal bovine serum and 12.5% horse serum was prepared as described in Example 1. For hematopoietic cultures HLTM was supplemented with 150 U/ml IL-3 (R&D Systems, Minneapolis, MN), 40 ng/ml IL-6 (Sandoz, East Hanover, NJ), 50 ng/ml SCF (Amgen, Thousand Oaks, CA), and 150 U/ml G-CSF (R&D Systems). Unless otherwise noted, all reagents were obtained from Sigma (St. Louis, MO).

Static cultures were performed as described in Koller M.R., et al., 1993, BioTechnol 11:358-363. Perfusion cultures were performed using the grooved bioreactor as described in

Example 1 above. The perfusion culture temperature was maintained at 37.0 ±0.5°C, and the pH and dissolved oxygen (DO) data acquisition and control systems were as described in Koller, et al (supra) with the exception that the pH was controlled by a gas mixing unit with separate ports for air, N_2, and CO_2. Nonadherent cells were retained through the use of rectangular grooves, which occupied one-half of the 30 cm^2 surface area, oriented perpendicular to the direction of flow (see Figs. 1-3). The pH was controlled at 7.35 ±0.05 by varying the ratio of CO_2 to air in the gas flow to the headspace in the medium holding tank. The DO in the medium outlet from the chambers never dropped below 90% of air saturation. Static cultures in 100 mm polycarbonate petri dishes (with spacers such that the surface area is 30 cm^2) were conducted at 37°C in a fully humidified incubator with an atmosphere of 5% CO_2 in air.

Perfusion and static cultures were initially seeded with either 2×10^6 mobilized peripheral blood MNCs or 2×10^5 $CD34^+$ cells (see Example 1 above). The initial cell densities were chosen to give approximately the same cell density on day 15. The initial medium volume was 120 ml (for 3 chambers) for perfusion cultures and 20 ml (each) for static cultures. The medium circulation rate in the perfusion system was gradually increased from 0 to 2.5 ml/minute/culture chamber over 1.5 hours. Negligible numbers of cells were observed in the cell trap at any time. Perfusion cultures were fed 3 times per week by replacing one-half of the medium with fresh HLTM and cytokines. After each chamber was harvested, the medium reservoir volume was decreased by 30 ml. Static cultures were fed every 5 days by replacing one-half of the medium with fresh HLTM and cytokines. The associated depopulation of nonadherent cells in the static cultures was 19 ±31%, as determined by cell counts on the medium removed.

One of three parallel cultures was sacrificed every 5 days to asses total cell numbers, cell viability, CFU-GM and LTC-IC content, cell phenotype and morphology as described below. In order to prevent enzymatic damage to the cells or cell surface markers, trypsin was not used to harvest the cultures. Perfusion and static cultures were harvested by removing the cell suspension from the culture chamber or petri dish, rinsing with 10 ml of Ca++ and Mg++ free phosphate buffered saline (CMF-PBS, Gibco, Grand Island, NY), rinsing with 10 ml cell dissociation solution (Sigma, #C-5789), and rinsing a second time with 10 ml CMF-PBS. The cells were then washed and resuspended in HLTM. Cell counts and viability were determined using a hemacytometer with trypan blue dye exclusion. The nonenzymatic harvest procedure recovered greater than 97% of total cells, as determined by rinsing the harvested culture chamber or petri dish with 10 ml cetrimide and counting the released nuclei on a Coulter Counter model MHR (Coulter Electronics, Hialeah, FL)(data not shown). In addition, microscopic examination of the culture chambers and the petri dishes after harvest revealed few remaining cells.

Morphology: Cytospin slides were prepared by centrifugation of 5,000-50,000 cells in cytospin funnels at 1,000 rpm for 5 minutes using a Shandon Cytospin™2 (Pittsburgh, PA). The cells were then stained with Wright-Giemsa stain (Harleco, Gibbstown, NJ) for 30 seconds, followed by a phosphate buffer rinse for 1 minute. The slides were then evaluated for the presence of blast cells, promyelocytes, myelocytes, metamyelocytes, banded and segmented neutrophils, megakaryocytes, and promonocytes and monocytes.

Colony assays were conducted as described in Example 1 above. The 0.8% methylcellulose colony assay medium was

supplemented with 150 U/ml IL-3, 40 IL-6, 200 U/ml granulocyte-macrophage CSF (GM-CSF, R&D Systems), 150 U/ml G-CSF and 10 U/ml erythropoietin (Epo, Amgen). Fresh and cultured MNCs were plated between 1,000 and 9,000 cells/ml, while fresh and cultured $CD34^+$ cells were plated between 500 and 4,500 cells/ml.

LTC-IC assays were conducted as described in Example 1 above. Fresh MNCs were plated between 2.5×10^4 and 2.0×10^5 cells per well and $CD34^+$ cells were plated between 1.25×10^4 and 1.0×10^5 cells per well on day 0. Cultured MNCs were plated in duplicate between 5.0×10^4 and 2.0×10^5 cells per well and cultured $CD34^+$ were plated between 2.5×10^4 and 1×10^5 cells per well.

Initial and cultured MNCs and $CD34^+$ cells were evaluated for CD33/CD34, CD11b/CD15 and CD11b/GlyA using a slight modification of a previously described method (Smith, S.L., et al., 1993, Exp. Hematol. 21:870-877). Briefly, the cells were labeled with the following combinations of monoclonal antibodies: PE-CD33 (Becton Dickinson)/FITC-CD34 (8G12, Baxter Immunotherapy Division), PE-CD11b (Becton Dickinson)/FITC-CD15 (Becton Dickinson) and PE-CD11b/FITC-GlyA (Amak, Inc., Westbrook, ME). The cells were than fixed with paraformaldehyde and subsequently quantitated with a FACScan™ flow cytometer. CD33 antigens are partially degraded by chymopapain used to release $CD34^+$ cells from the paramagnetic beads during $CD34^+$ cell selection.

The productivities of perfused MNC and $CD34^+$ cultures were compared by normalizing the results for each experiment to an initial sample containing 10^9 MNC. Normalized production of total cells, CFU-GM and LTC-IC were calculated for each

experiment by multiplying the results obtained in MNC and $CD34^+$ cultures by:

$$M_{MNC} = \frac{10^9\ MNCs}{X_{MNC}} \quad \text{or} \quad M_{CD34} = \frac{10^9\ MNCs}{X_{CD34}} \frac{\%CD34_{MNC}}{\%CD34_{CD34+}} Y_S$$

where M_{MNC} is the multiplier for the MNC culture, M_{CD34} is the multiplier for the $CD34^+$ cell culture, X_{MNC} is the initial number of MNCs per culture, X_{CD34} is the initial number of selected cells per $CD34^+$ cell culture. $\%CD34_{MNC}$ is the $\%CD34^+$ cells in the MNCs (before selection), $\%CD34_{CD34}$ is the % $CD34^+$ cells in the $CD34^+$ selected cells and Y_s is the yield of the selection process.

Data Analysis: Statistical analysis for comparison of total cells, CFU-GM and LTC-IC in perfused and static MNC and $CD34^+$ cultures was performed by taking the logarithm of the results and then using a paired Student's t-test. Statistical analysis for comparison of cell phenotypes in perfused and static MNC and $CD34^+$ cultures was conducted using a paired Student's t-test. Statistical analysis for comparison of total cells, CFU-GM and LTC-IC produced from the perfusion culture of an initial 10^9 MNC sample cultured as MNCs or $CD34^+$ cells was performed by taking the logarithm of the results and then using a paired Student's t-test. Data is reported as the mean ± standard deviation (S.D.).

Results are shown in Table 3 below:

Key for Table 3

PBMN Cells: peripheral blood mononuclear cells.

CD34+ Cells: CD34+ selected cells.

Bioreactor #1 and #2: perfusion culture using grooved bioreactor of the present invention.

Control #1 and #2: static culture.

Table 3

PBE#10 Summary	PBMNCells HLTM	MNC's vs CD34+ Cells	Cells	Source		
		150U/ml IL-3	average	UC+RT		
		40ng/ml IL-6	Initial CD34%	6.31%	Initial Viability	93.87%
		150U/ml G-CSF	Final CD34%	90.93%	Final Viability	98.09%
		50ng/ml SCF	Yield	50.63%		

Day	Cells	Culture	Hemacytometer Cells	± Cells	Colony Assay CFU-GM	± CFU-GM	LTC-IC Assay LTC-IC	± LTC-IC
0	PBMNCells		1,935,040	± 483,781	7,795	± 2,935	2,845	± 2,012
0	CD34+ Cells		203,687	± 46,699	9,584	± 6,792	3,310	± 2,279
5	PBMNCells	Bioreactor#1	1,513,861	± 726,084	70,314	± 58,622	2,275	± 2,412
5	CD34+ Cells	Bioreactor#2	509,778	± 280,095	51,039	± 35,621	1,887	± 2,467
5	PBMNCells	Control#1	1,447,819	± 729,428	74,685	± 59,506	1,955	± 3,164
5	CD34+ Cells	Control#2	369,444	± 404,597	40,818	± 45,331	1,152	± 2,046
10	PBMNCells	Bioreactor#1	4,938,042	± 4,499,822	142,641	±149,847	5,207	± 10,177
10	CD34+ Cells	Bioreactor#2	4,433,778	± 2,655,020	109,932	± 84,122	2,300	± 4,230
10	PBMNCells	Control#1	5,958,264	± 7,215,201	148,459	±183,070	2,531	± 5,316
10	CD34+ Cells	Control#2	2,323,167	± 2,839,436	84,941	±146,563	1,836	± 4,088
15	PBMNCells	Bioreactor#1	17,711,097	±18,580,123	150,577	±177,037	3,491	± 5,879
15	CD34+ Cells	Bioreactor#2	23,069,194	±13,334,187	175,167	±197,816	4,468	± 6,325
15	PBMNCells	Control#1	15,799,611	±16,621,915	125,940	±171,016	2,110	± 4,853
15	CD34+ Cells	Control#2	17,197,742	±13,830,103	107,004	±149,647	2,386	± 3,501

For the mononuclear cell cultures there was a delay ranging from 5-10 days before significant proliferation was observed. Average maximum expansions of 9.2- and 8.2-fold were obtained on day 15 in the perfusion and static cultures, respectively. Cell viability for perfused and static MNC cultures was 95(+/-5)% and 96(+/-5)%, respectively.

Perfusion and static MNC cultures gave similar expansion of CFU-GM over 15 days, with the maximum expansion seen on days 10 - 15. Maximum average CFU-GM expansions of 19-fold were obtained in both the perfusion and static cultures. The average fraction of cells giving rise to CFU-GM (0.4% on day zero) peaked on day 5 at 4.6% and 5.2% for the perfusion and static cultures, respectively. Expansion of BFU-E and CFU-Mix was observed in perfused and static cultures for only one of six experiments. In the other five experiments, small numbers of BFU-E were observed prior to day 10, and CFU-Mix were not observed beyond day 0.

Perfusion MNC cultures supported the primitive LTC-IC better than static cultures over 15 days. Perfusion expanded the average number of LTC-IC to 123% of the input number at day 15 (183% at day 10), although expansion was observed in only two of the experiments. In contrast, static cultures were only able to maintain LTC-IT at 74% of the input number on day 15 (89% at day 10), with expansion observed in only one of six experiments. The fraction of cells giving rise to LTC-IC fell off continuously for both culture types from an average of 0.15% on day 0 to 0.02% or less on day 15.

Perfusion cultured MNCs had a more primitive phenotype than the static cultured cells. Cells in perfusion maintained

Table 4

Table 4. Percent of cells from static and perfusion cultures of MNCs and $CD34^+$ cells expressing CD34 and CD33.

			% $CD34^+$						% $CD33^+$					
Culture	Cells	Day	Mean	±	S.D.	(Min.	-	Max.)	Mean	±	S.D.	(Min.	-	Max.)
	MNCs	0[a]	††6.31	±	3.86	(2.23	-	12.32)	64.06	±	27.92	(24.65	-	93.70)
	$CD34^+$	0[a]	††90.93	±	10.50	(70.76	-	97.54)	NA[c]					
Perfused	MNCs	5[b]	4.74	±	7.46	(0.25	-	13.35)	24.97	±	27.04	(2.53	-	54.99)
Static	MNCs	5[b]	5.96	±	10.09	(0.05	-	17.61)	48.61	±	42.62	(1.39	-	84.23)
Perfused	$CD34^+$	5[b]	**4.43	±	6.27	(0.38	-	11.65)	23.01	±	21.82	(1.55	-	45.17)
Static	$CD34^+$	5[b]	**3.45	±	4.14	(0.11	-	8.08)	51.39	±	45.09	(1.28	-	88.67)
Perfused	MNCs	10[a]	**0.94	±	1.30	(0.00	-	3.50)	36.29	±	34.14	(2.34	-	87.33)
Static	MNCs	10[a]	*1.20	±	2.07	(0.00	-	5.25)	49.08	±	39.78	(5.79	-	86.54)
Perfused	$CD34^+$	10[a]	**1.12	±	1.30	(0.03	-	3.00)	42.28	±	35.61	(3.13	-	86.80)
Static	$CD34^+$	10[a]	**0.29	±	0.52	(0.00	-	1.32)	32.87	±	33.96	(0.36	-	87.48)
Perfused	MNCs	15[a]	*,⊥0.59	±	0.56	(0.06	-	1.26)	46.01	±	38.82	(4.22	-	93.53)
Static	MNCs	15[a]	*,⊥0.03	±	0.03	(0.00	-	0.06)	38.19	±	31.97	(0.59	-	80.11)
Perfused	$CD34^+$	15[a]	**0.43	±	0.53	(0.05	-	1.21)	44.57	±	36.18	(8.41	-	94.32)
Static	$CD34^+$	15[a]	**0.04	±	0.09	(0.00	-	0.23)	40.41	±	33.65	(4.38	-	86.95)

[a] Average of six experiments.

[b] Average of three experiments.

[c] NA; not analyzed because CD33 antigen is partially sensitive to chymopapain, which is used in the selection process.

* and ** Differences from day 0 ($p < 0.05$ and $p < 0.01$, respectively).

⊥ and ⊥⊥ Differences between perfusion and static cultures ($p < 0.05$ and $p < 0.01$, respectively).

† and †† Differences between MNCs and $CD34^+$ cells ($p < 0.05$ and $p < 0.01$, respectively).

In addition, the fraction of $CD11b^-/CD15^-$ (immature cells) was consistently greater in perfusion, while the fraction of $CD11b^+/CD15^+$ (mature granulocytes) was consistently greater in static culture (Table 5).

TABLE 5

Table Percent of cells from static and perfusion cultures of MNCs and $CD34^+$ cells expressing $CD11b^-/CD15^-$ and $CD11b^+/CD15^+$.

			% $CD11b^-/CD15^-$		% $CD11b^+/CD15^+$	
Culture	Cells	Day	Mean ± S.D.	(Min. - Max.)	Mean ± S.D.	(Min. - Max.)
	MNCs	0[a]	††27.16 ± 20.64	(7.63 - 56.12)	††47.30 ± 24.26	(16.90 - 72.51)
	CD34+	0[a]	††87.05 ± 9.58	(74.03 - 97.16)	††4.29 ± 4.70	(0.71 - 13.00)
Perfused	MNCs	5[b]	⊥⊥47.72 ± 14.43	(33.18 - 62.04)	32.22 ± 17.32	(16.79 - 50.96)
Static	MNCs	5[b]	⊥⊥36.45 ± 12.57	(23.81 - 48.94)	36.07 ± 15.84	(20.54 - 52.21)
Perfused	CD34+	5[b]	69.92 ± 10.95	(59.06 - 80.95)	8.70 ± 4.27	(4.88 - 13.31)
Static	CD34+	5[b]	*57.68 ± 5.22	(54.23 - 63.68)	1.93 ± 0.75	(1.10 - 2.57)
Perfused	MNCs	10[a]	38.74 ± 15.55	(22.25 - 62.35)	30.56 ± 10.45	(17.55 - 47.96)
Static	MNCs	10[a]	25.13 ± 15.16	(9.16 - 48.50)	39.06 ± 15.88	(21.00 - 67.21)
Perfused	CD34+	10[a]	**43.13 ± 13.05	(25.90 - 61.19)	**23.59 ± 8.31	(9.33 - 33.57)
Static	CD34+	10[a]	**39.19 ± 18.98	(21.41 - 63.95)	**24.97 ± 14.15	(11.67 - 46.58)
Perfused	MNCs	15[a]	⊥19.43 ± 10.06	(8.28 - 37.15)	⊥58.29.± 14.11	(34.63 - 78.87)
Static	MNCs	15[a]	*,⊥6.47 ± 3.45	(2.74 - 11.13)	⊥70.55 ± 10.89	(59.24 - 91.10)
Perfused	CD34+	15[a]	**22.37 ± 13.42	(12.73 - 49.22)	**54.28 ± 14.74	(25.81 - 64.71)
Static	CD34+	15[a]	**20.22 ± 32.70	(5.70 - 86.92)	**59.37 ± 25.32	(7.86 - 72.50)

[a] Average of six experiments.

[b] Average of three experiments.

* and ** Differences from day 0 ($p < 0.05$ and $p < 0.01$, respectively).

⊥ and ⊥⊥ Differences between perfusion and static cultures ($p < 0.05$ and $p < 0.01$, respectively).

† and †† Differences between MNCs and $CD34^+$ cells ($p < 0.05$ and $p < 0.01$, respectively).

Less than 3% of the cells in perfusion or static culture expressed Glycophorin A (a red cell marker, data not shown). Importantly, the cells in MNC and CD34+ cell perfusion cultures were morphologically similar after 10 days despite significantly different initial compositions (Table 6, below).

Table 6

Table 6. Differential cell counts (%) on perfusion cultured MNCs and $CD34^+$ cells.[a]

Cells	Day	Blast Cells	Granulocytic Morphology Promyelo.[b]	Myelocytes	Metamyelo.	Band & Seg.	Total Gran.	Megakaryo.[c]	MO & Etc.
MNCs	0	$12 \pm 5^{**}$	$20 \pm 8^{**}$	$14 \pm 10^{*}$	$8 \pm 5^{*}$	2 ± 4	$45 \pm 8^{**}$	0	$43 \pm 10^{**}$
$CD34^+$	0	$83 \pm 11^{**}$	$0 \pm 0^{**}$	$2 \pm 3^{*}$	$1 \pm 1^{*}$	0 ± 0	$3 \pm 5^{**}$	0	$14 \pm 12^{**}$
MNCs	5	23 ± 23	$4 \pm 4^{\dagger}$	5 ± 5	4 ± 3	1 ± 1	$14 \pm 10^{\dagger\dagger}$	0	64 ± 29
$CD34^+$	5	48 ± 41	2 ± 3	$5 \pm 3^{\dagger}$	0 ± 1	0 ± 0	$8 \pm 3^{\dagger}$	0	44 ± 42
MNCs	10	9 ± 7	$35 \pm 13^{\dagger}$	16 ± 5	3 ± 1	1 ± 2	$55 \pm 9^{\dagger}$	0	35 ± 12
$CD34^+$	10	$4 \pm 3^{\dagger\dagger}$	$32 \pm 25^{\dagger}$	$18 \pm 11^{\dagger}$	6 ± 5	0 ± 1	$56 \pm 36^{\dagger}$	0	40 ± 38
MNCs	15	$0 \pm 0^{\dagger\dagger}$	23 ± 6	$24 \pm 8^{\dagger}$	18 ± 11	3 ± 4	$69 \pm 12^{\dagger\dagger}$	0	$33 \pm 11^{\dagger}$
$CD34^+$	15	$0 \pm 0^{\dagger\dagger}$	16 ± 14	$14 \pm 11^{\dagger}$	13 ± 15	3 ± 4	46 ± 39	0	54 ± 39

[a] Average of five experiments.

[b] Abbreviations: Promyelo., promyelocytes; Metamyelo., metamyelocytes; Band & Seg., band form and segmented neutrophils; Total Gran., Total cells with granulocytic morphology; Megakaryo., morphologically recognizable megakaryocytes; MO & Etc., macrophages, monocytes and morphologically unidentifiable cells (~ 10% of total cells).

[c] Morphologically, immature megakaryocytes are not distinguished from monocytes using Wright-Giemsa stain.

*, ** Significant differences between MNCs and $CD34^+$ cells ($p < 0.05$ and $p < 0.01$, respectively).

†, †† Significant differences between day 0 and indicated day ($p < 0.05$ and $p < 0.01$, respectively).

Perfusion and static $CD34^+$ cell cultures exhibited a continuous expansion of total cells over 15 days (Table 3, above).

Average maximum expansions of 113- and 84-fold were obtained on day 15 in the perfusion and static cultures, respectively. The lower average expansion in static culture is partially due to very low proliferation in one of the cultures. Interestingly, the static MNC culture from this sample also gave low expansion, while both perfusion cultures gave normal cell proliferation. Cell viability for perfused and static cultures was 96(+/-5)% and 92(+/-11)% respectively.

There was a trend toward larger expansion and better maintenance of CFU-GM numbers in the perfusion cultures. Maximum CFU-GM expansions of 18- and 11-fold were obtained on day 15 for the perfusion and static cultures, respectively. The fraction of cells giving rise to CFU-GM (4.7% on day 0) peaked on day 5 in the perfusion and static cultures at 10% and 11%, respectively. Expansion of BFU-E and CFU-Mix was observed in perfusion and static cultures for only one of six experiments. In the other five experiments small numbers of BFU-E were observed prior to day 15, and CFU-Mix were not observed beyond day 0.

Perfusion CD34+ cell cultures supported LTC-IC better than static cultures over 15 days. Perfusion expanded the average number of LTC-IC to 135% of the input value on day 15, although expansion was observed in only two experiments. In contrast, static cultures were only able to maintain LTC-IC at 72% of the input number on day 15, with expansion observed in only one of six experiments. The fraction of cells giving rise to LTC-IC decreased continuously for both culture types from an average of 1.6% on day 0 to 0.02% or less on day 15.

CD34+ cells in perfusion had similar phenotypes as the cells in static culture. Perfusion tended to maintain the CD34+ population longer, but the fraction of CD33+, CD11b-/CD15-, and CD11b/CD15+ cells were similar. On average, less than 3% of the cells in either culture expressed GlyA at any time. Initially, CD34+ cells were primarily blast cells. The fraction of blast cells decreased rapidly, and the cultures contained predominantly granulocytic and monocytic cells by day 10. Interestingly, the cultures that showed poor expansion of cells, CFU-GM, and LTC-IC contained primarily monocytic cells.

<u>Comparison of MNC and CD34$^+$ Cell Perfusion Cultures</u>:
We limit our comparison of the performance of cultures initiated with MNC and CD34$^+$ cells to perfused cultures. As noted above, the perfused cultures were generally equivalent to or better than the static cultures for samples that grew well. In addition, perfusion culture provided at least limited expansion of samples that failed to grow in the static cultures.

Although the MNCs and CD34$^+$ cells used to initiate the cultures had very different phenotypes, cells in the two perfusion culture systems expressed similar levels of CD34, CD33, CD11b$^+$/CD15$^+$, and CD11b$^-$/CD15$^-$ after 10 days of culture. The fraction of cells on day 15 in MNC and CD34$^+$ cell cultures, respectively, that was CD34$^+$ was 0.6% and 0.4%, while the fraction that was CD33$^+$ was 46% and 45%. In contrast, the populations used to seed these cultures were 6.3% and 90.9% CD34$^+$, respectively. The fraction of cells on day 15 in MNC and CD34$^+$ cell cultures, respectively, that was CD11b$^-$/CD15$^-$ (immature cells) was 19% and 22%, while the fraction that was CD11b$^+$/CD15$^+$ (mature granulocytes) was 58% and 54%. In contrast, the populations used to seed these cultures were 27% AND 87% CD11B$^-$/CD15$^-$ and 47% and 4%

$CD11b^+/CD15^+$, respectively. In addition, two-dimensional flow cytometry analysis of CD33/CD34 and CD11b/CD15 experession in a typical perfusion culture of MNCs and CD34+ cells revealed remarkably similar cell populations after 5 days.

Initially, the CD34+ cells contained predominantly blast cells with some monocytic cells and very few granulocytic cells, whereas the MNCs contained predominantly granulocytic and monocytic cells with some blast cells. However, after 10 days in perfusion, both cultures were predominantly granulocytic with a large fraction of monocytic cells. The prevalence towards the granulocytic lineage is greater than that indicated in Table 6 because: (1) mature granulocytes have short half-lives in culture even in the presence of G-CSF, (2) unidentifiable cells (about 10% of the total) are included with the monocytes, and (3) immature megakaryocytes are not distinguished from monocytes using Wright-Giemsa stain. Thus, perfusion cultures of PB MNCs and CD34+ cells appear to mature along the granulocytic lineage in a similar fashion for the growth factor combination used.

The fraction of cells giving rise to CFU-GM and LTC-IC in cultures initiated with MNCs and $CD34^+$ cells was also similar after 10 days of perfusion culture. The fraction of cells giving rise to CFU-GM was 2.9% and 2.5% on day 10 for MNCs and $CD34^+$ cells, respectively. This contrasts with 0.4% and 4.7% on day 0, respectively. The fraction of cells giving rise to LTC-IC was 0.10% and 0.043% on day 10 for MNCs and $CD34^+$ cells, respectively. Again, this is in contrast to 0.15% and 1.6% on day 0, respectively.

The total numbers of cells, CFU-GM and LTC-IC that could be obtained from perfusion culture of a peripheral blood

sample cultured as MNCs are greater than those that could be obtained for the same sample selected and cultured as $CD34^+$ cells (Table 7).

Table 7

Table 7. Viable cells. CFU-GM and LTC-IC that would be produced from an initial sample of 10^9 MB MNCs cultured in perfusion as either MNCs or $CD34^+$ cells.[a]

Cells	Day	Cells [10^6]	±	S.D.	(Min.	-	Max)
MNCs	0	1,000	±	0††	(1,000	-	1,000)
$CD34^+$	0	38	±	44††	(6	-	124)
MNCs	5	746	±	326†	(180	-	1,061)
$CD34^+$	5	147	±	231†	(6	-	609)
MNCs	10	2,661	±	2,630††	(726	-	7,551)
$CD34^+$	10	1,494	±	2,834††	(63	-	7,257)
MNCs	15	9,276	±	9,595	(1,821	-	23,405)
$CD34^+$	15	6,275	±	9,360	(292	-	24,774)

Cells	Day	CFU-GM [10^4]	±	S.D.	(Min.	-	Max)
MNCs	0	416	±	146†	(217	-	660)
$CD34^+$	0	165	±	175†	(5	-	509)
MNCs	5	3,629	±	2,994†	(976	-	8,191)
$CD34^+$	5	1,538	±	2,470†	(62	-	6,455)
MNCs	10	7,424	±	7,765††	(1,972	-	18,917)
$CD34^+$	10	3,039	±	5,634††	(130	-	14,427)
MNCs	15	7,650	±	8,571††	(1,834	-	24,426)
$CD34^+$	15	2,992	±	3,619††	(299	-	7,712)

Cells	Day	LTC-IC [10^3]	±	S.D.	(Min.	-	Max)
MNCs	0	1,532	±	1,109††	(579	-	3,235)
$CD34^+$	0	649	±	817††	(47	-	2,194)
MNCs	5	1,138	±	1,209††	(91	-	3,169)
$CD34^+$	5	467	±	721††	(13	-	1,758)
MNCs	10	2,609	±	4,943††	(65	-	12,587)
$CD34^+$	10	547	±	830††	(8	-	1,769)
MNCs	15	1,785	±	2,858	(17	-	7,408)
$CD34^+$	15	835	±	1,074	(25	-	2,319)

[a] Average of six experiments.

† and †† Differences between MNCs and $CD34^+$ cells ($p < 0.05$ and $p < 0.01$, respectively).

The maximum number of total cells, CFU-GM and LTC-IC were obtained on days 15, 10-15 and 10-15, respectively. Perfusion culture seeded with MNCs would yield 1.5-, 2.6- and 2.1-fold more total cells, CFU-GM and LTC-IC, respectively, on day 15 than would selecting and culturing the $CD34^+$ fraction, as determined using the culture performance, initial cell loading, and yield on the $CD34^+$ cell selection obtained for each experiment (see Data Analysis). Assuming a 100% $CD34^+$ selection yield for each of the experiments, production from MNCs on day 15 would be equivalent for total cells, 1.5- and 1.4-fold greater for CFU-GM and LTC-IC respectively than from $CD34^+$ cells, although these differences would not be significant. Harvesting on day 10 may be optimal because CFU-GM numbers, which are an indicator of transplant quality, are essentially unchanged between days 10 and 15, while the fraction of total cells that give rise to CFU-GM is more than 3-times greater on day 10. In addition, the number of LTC-IC in MNC cultures was greater on Day 10 than day 15 in three of six experiments, and equivalent in two others. For harvest on day 10, perfusion cultures seeded with MNCs would produce 1.8, 2.4-, and 4.8-fold more total cells, CFU-GM, and LTC-IC, respectively, than those seeded with $CD34^+$ cells.

DISCUSSION

Expansion of total cells and CFU-GM was obtained in static and perfused cultures initiated with either peripheral blood MNCs or $CD34^+$ cells. Perfusion CD34+ cell cultures and perfusion MNC cultures supported LTC-IC better than static cultures over 15 days.

Other investigators have examined the large scale culture of either MNCs or $CD34^+$ cells for expansion of CFU-GM in tissue culture flasks (McAlister IB, et al., Exp Hematol

20:626-628, 1992; Haylock DN, et al., Blood 80:1405-1412, 1992; Sato, N., et al., Blood 82:3600-3609, 1993; Brugger, W., et al., Blood 81:2579-2584, 1993), gas permeable culture bags (Takaue Y, et al., Ann Hematol 64:217-220, 1992; Lemoli RM, et al., Exp Hematol 20:259-275, 1992) and perfusion systems (Koller, MR., et al., Bio/Technol 11:358-363, 1993; Palsson, BO., et al., Bio/Technol 11:368-372, 1993; Koller, MR., et al., Blood 82:378-384, 1993). In most of these studies the maximum CFU-GM expansion was found between days 7 and 14. The 19-fold maximum CFU-GM expansion obtained for MNC cultures compares favorably to the 3.8- to 16-fold expansion reported for peripheral blood MNCs (PBMNCs)(Takaue et al., supra; McAlister, et al., supra). In contrast, the 11- to 18-fold maximum CFU-GM expansion for $CD34^+$ cell cultures is lower than previously reported 57- to 190-fold expansions for PB $CD34^+$ cells (Haylock DN, et al., supra; Sato N, et al., supra; Brugger W., et al., supra). However, these large CFU-GM expansions were obtained using combinations of five or six growth factors, while expansion of CFU-GM in MNC cultures was obtained using combinations of two or three growth factors. For example, Haylock et al, reported a maximum CFU-GM expansion of 66-fold after 14 days of PB $CD34^+$ cell culture with IL-1β, IL-3, IL-6, G-CSF, GM-CSF and SCF. They report that, after 7 days of culture, this six growth factor combination gave the maximum CFU-GM expansion of 31 different growth factor combinations analyzed. Their optimal combination gave approximately 5-fold more CFU-GM than the three factor combination of IL-3, GM-CSF and SCF used by McAlister et al. (actually PIXY321 (GM-CSF and IL-3 fusion protein) and SCF). Assuming this ratio remained the same for a 14 day culture, the maximum CFU-GM expansion would decrease from 66-fold to 13-fold, which is similar to the 16-fold expansion reported by McAlister et ala. Similarly, Sato et al. reported a 57-fold expansion of CFU-

GM from highly-purified PB $CD34^+$ cells after 7 days in culture with IL-3, IL-6, G-CSF, GM-CSF, and SCF. However, expansion was only 25-fold with IL-3, IL-6, G-CSF, and SCF, which is similar to CFU-GM expansions obtained in the present study using the same cytokines. Brugger et al. report a 190-fold expansion of CFU-GM in PB $CD34^+$ cell cultures with IL-1, IL-3, IL-6, Epo, and SCF, but obtained only 20- to 40-fold expansions when G-CSF was also used, as well as for a variety of 4-factor cytokine combinations.

Differences between results reported by various investigators for $CD34^+$ cell cultures may also be due to differences in the feeding protocol (e.g., how depopulation is accounted for), $CD34^+$ cell selection methods and culture media used, and sample sources. With regard to the latter item, PB samples were used from normal donors and cancer patients mobilized with chemotherapy and/or growth factor regimens. Peripheral blood from these sources can vary greatly in the fraction of primitive cells. For example, Brugger et al, indicate that only 0.2% of the $CD34^+$ cells obtained from chemotherapy and G-CSF mobilized blood formed CFU-GM colonies. This contrasts with 5% of $CD34^+$ cells obtained from cyclophosphamide and G-CSF mobilized blood (this study) and 7.6% of $CD34^+$ cells from normal blood (Sato et al., supra). Given the large number of factors that alter cell expansion, it is best to directly compare the effects of any particular parameter using the same cell source and protocol.

Interestingly, using the method of the present invention, cultures inoculated with either MNCs or $CD34^+$ cells produced cells that were remarkably similar after 10 days of culture. Changes observed in cell phenotype followed similar patterns of myeloid differentiation reported for cultures of bone marrow (Smith SL, et al., Exp Hematol

21:870-877, 1993) and cord blood (Terstappen LWMM, et al., Leukemia 6:1001-1010, 1992). During the early stages of myeloid differentiation, CD34$^+$ cells gain CD33 and lose CD34. The cells can further differentiate, with those maturing towards neutrophils acquiring CD15 followed by CD11b, while those maturing towards monocytes acquire CD11b and then CD15. This suggests that the CFU-GM present in expanded cell populations may be more mature than those present in uncultured cells. Infusion of large numbers of mature progenitor cells has the potential to decrease the extent and duration of cytopenias following transplantation.

Due to the similar cell populations produced, the MNC and CD34$^+$ cell perfusion cultures can be compared directly in terms of the quantity of cells, CFU-GM and LTC-IC produced. After 15 days in perfusion culture, MNCs produced 1.5-,2.6- and 2.1-fold more total cells, CFU-GM and LTC-IC, respectively, than would the same sample selected and cultured as CD34$^+$ cells. Even if the CD34 selection process was 100% efficient, production of CFU-GM would be 1.5-fold greater for MNCs than for CD34$^+$ cells. This difference does not appear to be due to losses incurred during the selection process because when the yield on the CD34 selection is considered, 100(± 100)% of the CFU-GM and 70(±30)% of the LTC-IC are recovered. While production of CFU-GM from MNC cultures may not exceed that from CD34$^+$ cell cultures for all initial cell populations and culture conditions, our results clearly demonstrate that selection of CD34$^+$ cells is not required in order to obtain extensive CFU-GM expansion.

CD34 cell selection may still be desirable for reasons other than increasing cell expansion. Recently, it has been shown that tumor cells in breast (Ross AA, et al.,

Blood 82:2605-2610, 1993) and small cell lung cancer (Brugger W, et al., Blood 83:646-640, 1994) can be mobilized into the peripheral blood along with hematopoietic progenitor cells. Based on the selective loss of leukemic cells in bone marrow cell culture (Da WM, et al., Brit J Hematol 78:42-47, 1991; Testa NG, et al., Hematol Blut Transfus 31:75-78, 1987; Barnett MJ, et al., Bone Marrow Transplant 4:345-351, 1989) *ex vivo* culture may also be expected to deplete nonhematopoietic tumor cells. However, selection of $CD34^+$ cells may still be required to provide additional purging. While stem cells *per se* are not required for reconstitution following myelosuppressive therapy, the decrease in LTC-IC numbers during mobilized blood culture may adversely affect long-term reconstitution following myeloablative therapy. Under these circumstances, it may be best to combine expanded cells (to provide large numbers of mature progenitors) with uncultured cells. In this regard, CD34 selection reduces the total volume for transplantation using uncultured cells and modulates graft vs. host disease in allotransplants. Finally, cultured $CD34^+$ cells may increase the efficiency of transfection for gene therapy.

Static and perfusion cultures gave similar average total cell and CFU-GM expansions for both MNC and $CD34^+$ cell cultures. In fact, when cell depopulation during feeding is taken into account, the static cultures would give a greater average expansion of total cells for both types and of CFU-GM for MNC cultures. However, it must be remembered that correcting for cell depopulation assumes that (1) the cells removed represent a uniform cell sampling, (2) these cells would either be returned to the culture or seeded into additional cultures, and (3) returning the cells would not affect the culture performance. Similar total cell and CFU-GM production in static and perfused PB MNC cultures

contrasts with results for cord blood MNCs (CB MNCs) cultured on irradiated stroma. In the latter case total cell production was greater and CFU-GM expansion was twice as great in perfusion culture compared to that in static culture (Koller et al., 1993, supra). We have also observed increased production of total cells and CFU-GM in perfused vs. static cultures of PB MNCs cultured on irradiated stroma (see Example 1 above). Better relative performance for the static cultures in the absence of stromal cells could be due to lower metabolic requirements of MNCs compared to stromal cells. However, it should be noted that we saw no relative decrease in the performance of static cultures for PB samples that exhibited greater cell expansion. In fact, a major advantage of perfusion culture is that those samples that performed very poorly in static culture exhibited at least limited (and in most cases normal) expansion in perfusion. In addition, perfusion cultures maintained LTC-IC numbers better than the static cultures, which is consistent with results for PB and CB MNCs on irradiated stroma (Koller,et al., 1993, supra).

Perfused bioreactors are superior to bag or flask cultures for progenitor cell expansion for transplantation because they maintain desired culture condition, minimize chances for contamination during feeding, are easier to scale up for clinical application, and facilitate compliance with current and expected Food and Drug Administration (FDA) regulations. In 1992 the FDA stated that CFR section 211 (21 CFR 211), the set of regulations known commonly as GMP, is legally applicable to blood handling establishments. CFR 211.22 mandates the institution of a quality control unit, the head of which must be distinct from the transfusion center director. Increased FDA regulatory activity is anticipated for cellular therapies such as

autolymphocyte therapy and bone marrow transplant. Ex vivo expansion of hematopoietic cells will most certainly be governed by 21 CFR 211. In complying with GMP regulations, representative samples of material in-process are needed to monitor quality, while at the same time a closed culture system is highly desirable. Thus, a perfusion bioreactor designed with the ability to draw samples is especially suitable for processing under GMP regulations. The greatest advantage for bioreactor systems, however, lies in the area of validation. Validation, a requirement implicit in 21 CFR 211.100, consists of establishing documented evidence that the process in question consistently and reproducibly provides a product of predetermined quality and specification.

The results obtained for total cell and CFU-GM expansion can be used to estimate the size of the initial mobilized blood sample and culture system required for therapeutic application of cultured hematopoietic cells. A therapeutic dose of 20 x 10^4 CFU-GM/kg body weight has been suggested for rapid engraftment of neutrophils using peripheral blood cells (Bender JG, et al., J Hematotherapy 1:329-341, 1992). An 80 kg individual would then require 16 x 10^6 CFU-GM. Either 3.8 x 10^9 uncultured mobilized blood MNCs or 0.21 x 10^9 MNC cultured for 15 day with IL-3, IL-6, G-CSF and SCR would be required for 16 x 10^6 CFU-GM. The culture system would have to accommodate at least 2 x 10^9 cultured MNCs. Since neither culture exhibited indications of limiting cell proliferation due to cell density, an estimate for the maximum cell density obtainable for the perfusion and static cultures can be found by dividing the maximum cell numbers obtained per culture by the culture surface area. The maximum obtained in perfusion was 48 x 10^6 cells on effectively 15 cm^2 culture area, or 3.2 x 10^6 cells/cm^2 assuming that the cells are only in the grooves. The

maximum obtained in static culture was 45 x 10^6 cells on 30 cm^2 culture area, or 1.5 x 10^6 cells/cm^2. Under these conditions, the perfusion system would require 625 cm^2, while the static culture would require 1350 cm^2 (or the equivalent of six T-250 culture flasks). To obtain an upper limit, similar calculations can be performed for the sample that exhibited the lowest fraction of CFU-GM on day 15 in perfusion culture. This gives an estimate of 1500 cm^2 and 15,000 cm^2 for the perfusion and static cultures, respectively.

Example 3

<u>Cord Blood Mononuclear Cells in Smooth versus Grooved Perfusion Chambers.</u>

Suspensions of cord blood (CB) mononuclear cells were prepared as described in Example 1. Cytokine concentrations for all cultures were as in Example 1. Perfusion and static cultures were conducted as in Example 2. Culture medium was HLTM as in Example 1, containing 12.5% preselected lots of FBS and horse serum, respectively. Stroma-free CB MNC cultures, supplemented with IL-3, IL-6, G-CSF, and SCF, were conducted in both smooth perfusion culture chambers and the grooved bioreactor of the present invention. Control static cultures were conducted in petri dishes. No stroma was used in this series of experiments.

Results are shown in Tables 8 and 9 below:

Key for Table 8 and 9:

CD MNCs: cord blood mononuclear cells

Smooth: perfusion culture, smooth-bottom chamber, no stroma (Koller, et al., 1993, <u>supra</u>).

Grooved: perfusion culture using the grooved bioreactor of the present invention, no stroma.

Control: static culture in petri dish, no stroma.

Table 8

Table 8
PBE #11 Sum. Smooth vs. Grooved Chamber Feeding Schedule
HLTM 150 U/ml IL-3 (R&D) Bioreactor - 1/2 medium 3 times per week
B1 Smooth 40 ng/ml IL-6 (R&D Systems) Control - 1/2 medium every 5 days
B2 Grooved 150 U/ml G-CSF (Immunex)
50 ng/ml SCF (Amgen)

		Hemacytometer		Hemacytometer	
Day	Culture	Cells	± Cells	Viability	± Viability
0	CB MNCs	5,150,000	± 94,281	96%	± 2%
5	Smooth	2,013,125	± 188,267	74%	± 12%
5	Grooved	2,621,750	± 602,101	74%	± 4%
5	Control	2,831,458	± 425,384	91%	± 5%
10	Smooth	7,038,750	± 138,946	88%	± 4%
10	Grooved	10,327,375	± 3,604,300	93%	± 5%
10	Control	8,303,333	± 7,129,993	84%	± 18%
15	Smooth	15,512,667	± 5,744,535	93%	± 3%
15	Grooved	36,819,375	± 6,421,413	97%	± 3%
15	Control	32,307,750	± 4,254,308	93%	± 3%

Table 9

Day	Culture	CFU-GM	± CFU-GM	BFU-E	± BFU-E	CFU-Mix	± CFU-Mix	CFU-c	± CFU-c
0	CB MNCs	25,927	± 4,956	4,244	± 584	14,610	± 7,383	44,781	± 12,924
5	Smooth	82,169	± 5,154	53,966	± 4,305	70,978	± 24,363	207,113	± 23,514
5	Grooved	132,088	± 24,901	64,774	± 9,576	90,173	± 27,900	287,035	± 6,577
5	Control	132,471	± 36,720	85,431	± 7,851	98,229	± 845	316,131	± 45,417
10	Smooth	208,723	± 124,325	78,778	± 95,816	52,987	± 71,639	340,487	± 291,780
10	Grooved	336,595	± 57,421	130,736	± 145,582	65,406	± 67,229	532,736	± 155,390
10	Control	239,501	± 216,526	59,508	± 27,526	40,305	± 14,532	339,314	± 174,467
15	Smooth	262,792	± 223,847	7,378	± 8,903	3,668	± 4,877	273,838	± 237,626
15	Grooved	571,789	± 155,704	29,239	± 25,042	5,155	± 2,930	606,182	± 183,676
15	Control	335,849	± 5,096	61,251	± 77,327	32,087	± 43,513	429,187	± 115,745

Perfusion cultures in the grooved bioreactor showed similar cell expansion as the cultures in the static cultures. However, perfusion cultures in the smooth chamber showed only one-half the cell expansion as perfusion cultures in the grooved bioreactor. Few, if any, cells were washed out of the grooved chamber as evidences by few, if any, cells being found in the cell trap after the grooved, but not the smooth, chamber. Viability was below 20% for the cells found in the cell trap.

Perfusion cultures in the grooved chamber gave greater CFU-GM, BFU-E, and CFU-Mix expansion than static cultures in petri dishes. Maximum CFU-GM expansion of 22-, 10-, and 13-fold were obtained from cultures in the grooved chamber, smooth chamber, and petri dish respectively, on Day 10. Furthermore, maximum CFU-Mix expansion of 6.2-, 4.9, and 6.7-fold were obtained from cultures in the grooved chamber, smooth chamber, and petri dish, respectively, on day 5. This resulted in the distribution of colony types from the three cultures having 57% erythroid containing colonies on day 5 (e.g. BFU-E and CFU-Mix), 35% erythroid containing colonies on day 10, and only 10% erythroid containing colonies on day 15. Finally, the smooth chamber did not appear to preferentially retain specific cells over others as evidenced by the similar distribution of colony types and the fraction of cells giving rise to CFU-C in the cultures with grooved and smooth chambers.

WHAT IS CLAIMED IS:

1. A flow-through bioreactor for the retention and culture of cells in perfused media, said bioreactor comprising;

a generally rectangular vessel having a longitudinal axis, said vessel having a lid and a bottom wall connected to side walls and end walls,

said lid having an inlet port connected to an inlet slot, and an outlet slot connected to an outlet port, said inlet and outlet slots being positioned at opposite ends of said lid to allow for media flow along the longitudinal axis of said vessel,

said bottom wall having an inner surface, said inner surface having a plurality of generally rectangular grooves having a width, a depth, and a length, said grooves being positioned such that their length is transverse to the longitudinal axis of said vessel.

2. The flow-through bioreactor of claim 1 wherein said grooves have a width to depth ratio of about 1:1.

3. The flow-through bioreactor of claim 2 wherein said grooves have a width of about 50μm to about 5,000μm and a depth of about 50μm to about 5,000μm.

4. The flow-through bioreactor of claim 3 wherein said grooves have a width of about 200μm and a depth of about 200μm.

5. The flow-through bioreactor of claim 1 wherein said grooves have a width to depth ratio of about 2:1.

6. A method for culturing hematopoietic cells, comprising

placing a suspension of said cells in a bioreactor according to any one of claims 1-5, and culturing said cells in media perfused through said bioreactor.

7. The method of claim 6 wherein said suspension of cells comprises hematopoietic mononuclear cells, unselected for CD34+ cells.

8. The method of claim 6 wherein said suspension of cells comprises hematopoietic stem/progenitor cells, selected for CD34+ cells.

FIG. 1.

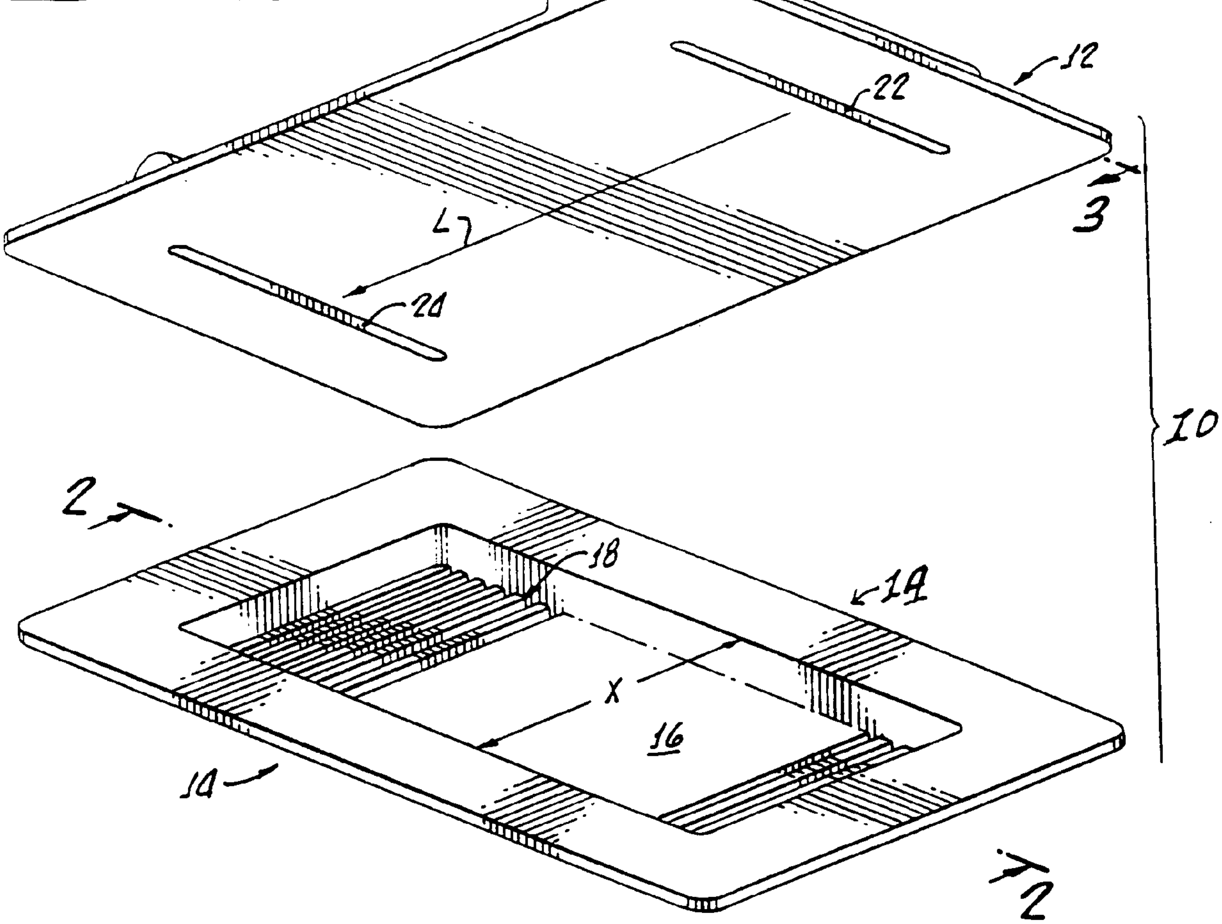


FIG. 2.

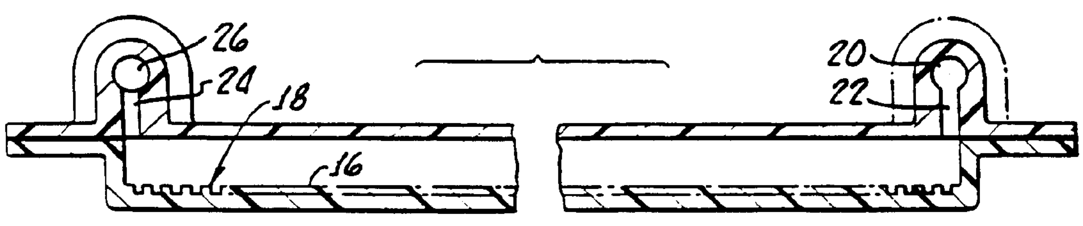


FIG. 3.

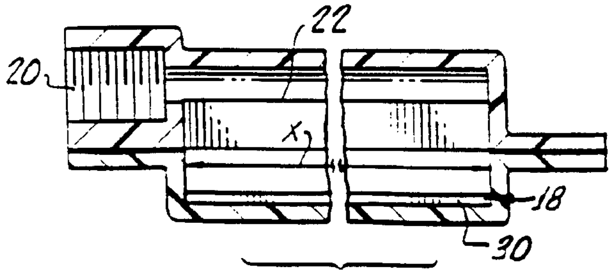


FIG. 4a.

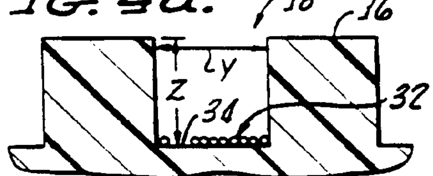
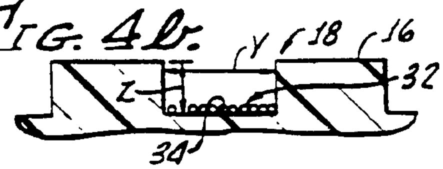


FIG. 4b.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02686

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12M 3/00, 3/04; C12N 5/00, 5/06, 5/12, 5/16
US CL : 435/240.2, 240.21, 284, 287, 296, 813
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.21, 284, 287, 296, 813

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: rectangular groove, corrugat, cell culture, receptacle, ridges, roller bottle

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A, 5,240,854 (BERRY ET AL) 31 August 1993, see entire document, especially col 2 lines 53+ and col 5 lines 49+.	1-5
Y	US,A, 5,010,013 (SERKES ET AL) 23 April 1991, see entire document.	1-5
Y	Bio/Technology, Volume 11, issued 11 March 1993, Koller M.R. et al, "Expansion of Primitive Human Hematopoietic Progenitors in a Perfusion Bioreactor System with IL-3, IL-6, and Stem Cell Factor", pages 358-363, see entire document.	6-8
Y	Experimental Hematology, Volume 21, issued 1993, Smith S.L. et al, "Expansion of Neutrophil Precursors and Progenitors in Suspension Cultures of CD34+ Cells Enriched from Human Bone Marrow", pages 870-877, see entire document.	6-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 APRIL 1995	31 MAY 1995

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